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Prevalence of Drug Resistance Mutations and HIV Type 1 Subtypes in an HIV Type 1-Infected Cohort in Rural Tanzania

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Abstract

The development of resistance mutations in drug-targeted HIV-1 genes compromises the success of antiretroviral therapy (ART) programs. Genotyping of these mutations enables adjusted therapeutic decisions both at the individual and population level. We investigated over time the prevalence of HIV-1 primary drug resistance mutations in treatment-naive patients and described the HIV-1 subtype distribution in a cohort in rural Tanzania at the beginning of the ART rollout in 2005–2007 and later in 2009. Viral RNA was analyzed in 387 baseline plasma samples from treatment-naive patients over a period of 5 years. The reverse transcriptase (RT) and protease genes were reversely transcribed, polymerase chain reaction (PCR) amplified, and directly sequenced to identify HIV-1 subtypes and single nucleotide polymorphisms associated with drug resistance (DR-SNPs). The prevalence of major DR-SNPs in 2005–2007 in the RT gene was determined: K103N (5.0%), Y181C (2.5%), M184V (2.5%), and G190A (1.7%), and M41L, K65KR, K70KR, and L74LV (0.8%). In samples from 2009 only K103N (3.3%), M184V, and T215FY (0.8%) were detected. Initial frequencies of subtypes C, A, D, and recombinants were 43%, 32%, 18%, and 7%, respectively. Later similar frequencies were found except for the recombinants, which were found twice as often (15%), highlighting the subtype diversity and a relatively stable subtype frequency in the area. DR-SNPs were found at initiation of the cohort despite very low previous ART use in the area. Statistically, frequencies of major mutations did not change significantly over the studied 5-year interval. These mutations could reflect primary resistances and may indicate a possible risk for treatment failure.

Introduction

It is universally recognized that combined antiretroviral therapy (ART) has dramatically reduced HIV-related mortality worldwide.[1,2] However, one major concern is that a rapid and not appropriately controlled scaling up of ART may accelerate the selection of drug resistance-associated mutations and transmission of HIV drug-resistant strains in a given population. This could impair basic ART programs as well as strategies for reducing HIV morbidity and mortality.[3]

The genetic diversity among HIV-1 subtypes is extensive. Based on data from the Los Alamos database the median percentage of amino acid differences within a given subtype was found to be 17% in Env and 8% in Gag, whereas intersubtype differences were 25% and 17%, respectively.[4] In contrast to the highly variable Env, the pol sequence is more conserved.[4,5] Different HIV-1 subtypes exhibit differences in frequency and route of virus transmission, in the pathogenesis of the disease as well as in the kinetics and mechanisms of drug resistance development, thus potentially affecting HIV-1 disease management.[3,6,7] In view of the above, we aimed to investigate key resistance mutations in the reverse transcriptase (RT) and protease genes following ART rollout in a rural setting in Ifakara, Tanzania.

In Tanzania, the first cases of HIV-1 infection were observed and reported in the Kagera region in 1983,[8] and by 2009 HIV prevalence in adults between 15 and 49 years of age

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was estimated to be 5.7% (6.6% women, 4.6% men), corresponding to 1.5 million infected people. The National ART program in Tanzania started in 2004.[9] The default first line drugs in Tanzania were zidovudine/stavudine, lamivudine, and nevirapine/efavirenz, and the second line drugs were abacavir, didanosine, and lopinavir/ritonavir or indinavir/ritonavir. By 2009 more than 454,000 Tanzanian HIV-1 patients were in need of ART (according to the 2002 WHO criteria for the initiation of ART) whereas only about 235,000 patients received ART.[10] However, according to the more recent WHO criteria for initiation of ART published in 2010[11] only one-third of the eligible patients in Tanzania were actually on ART. With many stakeholders supporting ART in the country, the Ministry of Health and Social Welfare (MoHSW) of Tanzania currently works on further expanding ART coverage through its National Aids Control Programme (NACP).

Studies on the transmission of primary HIV-1 drug resistance in Tanzania are few and limited to urban areas with large HIV-1 sentinel centers, e.g., Mbeya, Kagera, Kilimanjaro, and Dar es Salaam.[12,13] Corresponding data about the situation in rural areas are needed, as 80% of Tanzanians live outside the urban centers, and these surveillance data will add to a more precise and reliable picture of the HIV drug resistance situation in Tanzania.

In late 2004 an HIV cohort was established in Morogoro, rural Tanzania. The aim of this Kilombero-Ulanga-Antiretroviral-Cohort (KIULARCO) was to implement the care and treatment of HIV/AIDS patients according to Tanzania NACP guidelines, to strengthen infrastructure, to provide education of staff, to conduct research on optimal strategies for delivering treatment, and to conduct follow-up care in a resource-limited and rural setting in Tanzania.[4,14]

To date, a total of 5,748 HIV-infected individuals have been enrolled at the Chronic Disease Center of Ifakara (CDCI). After a patient is enrolled, biomedical data are collected longitudinally. Blood samples are routinely collected at enrollment, before initiation of ART, and at different time points during visits to the clinic. Clinical and immunological parameters of patients under ART are assessed routinely during follow-up visits. Details on the population and structure of the KIULARCO cohort have been described previously.[14,15]

This study aimed to investigate the prevalence of HIV-1 drug resistance mutations in treatment-naive patients and to establish HIV-1 subtypes in the KIULARCO cohort in Ifakara Tanzania between 2005–2007 and 2009.

Materials and Methods

Ethical considerations

The KIULARCO study was approved by ethics review bodies of Tanzania, the Ifakara Health Institute (IHI) Institutional Review Board, the National Institute for Medical Research (NIMR), and the Ethics Committee of the University and State of Basel (EKBB). Patients were asked for their consent before participation in this study.

Study site and subjects

Patients were enrolled at the CDCI, which serves as a Care and Treatment Center for HIV/AIDS patients and is affiliated with the Ifakara Health Institute (IHI) and St. Francis Referral Hospital (SFRH). The SFRH is an important health facility in the Kilombero and Ulanga districts in Morogoro, southeast Tanzania, and serves a population of about 600,000 individuals. It is estimated that more than 30,000 individuals infected with HIV-1 live in the area.[14] By July 2008, a total of 2,394 patients had been enrolled in the CDCI.[14,15] As of October 2011, the cumulative number of patients enrolled in CDCI was 5,748, of which 3,664 (63.7%) were females and 2,084 (36.3%) were males.

Depending on their CD4 counts and HIV-1 WHO clinical staging, participants either received ART (CD4 cell counts ≤ 200 cells/μl regardless of WHO stage, CD4 counts ≤ 350 cells/μl and WHO clinical stage 3, or WHO stage 4 regardless of CD4 cell count) or continued to be regularly followed up every 3 months. Before ART initiation, patient blood was drawn and plasma prepared and stored at -80°C. Data on clinical, virological, and immunological parameters, and demographic data of the patients (i.e., CD4 count, full blood picture, viral load, WHO clinical staging at enrollment, age, sex, and home town or village) were collected.

A total of 187 plasma samples from the time period 2005–2007 were randomly selected from all baseline samples collected prior to initiation of ART. Of those samples, 137 were derived from patients starting ART immediately after this test sample was collected. Fifty samples were chosen from HIV-1-infected patients, who were not yet eligible for initiation of ART. A second survey was conducted in 200 patients enrolled in the cohort in 2009. The 2009 inclusion criteria were HIV-1-positive patients, age >18 years, and recent CD4 counts >250 cells/μl (WHO clinical stage 1 or 2) or >350 cells/μl (WHO clinical stage 3). All plasma aliquots were sent to Basel, Switzerland, for molecular genotyping.

RNA extraction, RT-PCR, PCR, and sequencing

Viral RNA was extracted from plasma with either the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or the Macherey-Nagel NucleoSpin RNA Virus Kit (Macherey-Nagel GmbH & Co KG, Neumann-Neander, Germany) using the manufacturer's protocol.

RT was performed using specific primer RT2 (5′-GATAAGCTTGGGCCTTATCTATTCCAT-3′), AffinityScript RT Buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$), 2 μl of 100 mM DTT (Stratagene, La Jolla, CA), 0.8 μl dNTP mix (25 mM each dNTP), 1 μl of an RNase inhibitor, RNase Out (40 U/ μl), 1 μl AffinityScript Multiple Temperature Reverse Transcriptase, 1 μl specific Primer RT2 (10 μM), HPLC purified, and 9.5 μl RNA sample. RT was performed with the following thermal conditions: 42°C for 35 min, 55°C for 25 min, 70°C for 15 min, and 5°C for 15 min.

Primary polymerase chain reaction (pPCR) was done using Advantage cDNA Polymerase according to the supplier's protocol (Clontech Laboratories Inc., Mountain View, CA) with some modifications. Reverse and forward primers RT2 and D1818 (5′-AGAAGAAATGATGACAGCATGTCAGGGAGT-3′) were used. The pPCR mix contained 5 μl 10$\times$ Advantage buffer (Clontech), 10 μl dNTP mix (2 mM), 2 μl reverse primer RT2 (10 μM), 2 μl forward primer D1818 (10 μM), 1 μl Advantage Polymerase (5 U/μl), and 4 μl of cDNA. The reaction profile was 94°C for 2 min, 94°C for 20 s, 47°C for 20 s, and 68°C for 2 min; 30 cycles and a final elongation step at 68°C for 5 min were performed.

The nested PCR (nPCR) mix for amplification of the HIV-1 reverse transcriptase gene was 5 μl 10$\times$ Pfu buffer (Promega

Corporation, Madison, WI), 10 μl dNTP mix (2 mM), 2 μl forward primer JG103 5′-AACAATggCCATTgACAgAA[I-Q]-3′ (10 μM), 2 μl reverse primer JG202 5′-TCAggATggAgTTCATAICCCA-3′ (10 μM), 0.7 μl FIREPol Polymerase (3 U/μl), 0.1 μl Pfu Polymerase (3 U/μl), and 2 μl pPCR product. The thermocycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 47°C for 15 s, 72°C for 2 min, and a final elongation step of 72°C for 5 min. PCR conditions for amplification of the protease gene were the same as for the RT gene except that a different primer set was used: forward primer D2213A2 (5′-AGCAGGATCCGAAAGACAGGGA-3′) (10 μM) and reverse primer R2598L (5′-CCATCCCGGGCTTTAATTT TACTGG-3′) (10 μM). The nPCR products were purified with the NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's protocol.

Direct sequencing of purified nPCR products was performed either in house or by the commercial supplier Macrogen, South Korea. The in-house protocol used either one of the forward primers JG103 or PMF (5′-AACTCAAGACTTT TGGGAAGT-3′) or one of the reverse primers JG202 or PMR (5′-TTGTCATGCTACTCTGGAATA-3′). PMF and PMR are centrally located sequencing primers for the RT gene. For nested PCR amplification and sequencing of the protease gene, the reagents and protocol used were the same as above with the exception that forward primer D2213A2 and reverse primer R2598L were used instead of RT-specific nested primers. The sequences obtained for each sample were aligned using the Seqscape Software Programme Version 2.6 (AB, Applied Biosystems, Foster City, CA). The consensus sequences were assessed for drug resistance mutations by using the Stanford University HIV Drug Resistance Database HIVdb program Version 6.2.0.

HIV-1 subtyping

HIV-1 subtype information was obtained after submitting the sequences to the Los Alamos subtyping tool and by constructing phylogenetic trees using the maximum likelihood method based on the Tamura–Nei model. A total of nine HIV-1 reference subtypes and one simian deficiency virus were included in the phylogenetic analysis: subtype A1 (U51190, DQ676872, AF004885), subtype B (K03455), subtype C (U46016, U52953), subtype D (U88822, M27323, K03454), and simian immunodeficiency virus (U42720).

Viral load determination

Viral load was determined with a One Step Real-Time PCR System (Applied Biosystems) by using a modification of the manufacturer's instructions. cDNA was synthesized as shown above, but using random primers (0.1 μg/μl). The cDNA was then quantified by qRT-PCR. The qRT-PCR reaction contained 12.5 μl TaqMan Gene Expression Master Mix, 0.125 μl forward primer M2227F, 5′-AGC CTC AAT AAA GCT TGC CTT G-3′ (10 μM), 0.125 μl reverse primer M2228R, 5′-CGG GCG CCA CTG CTA G-3′ (10 μM), 0.5 μl of probe HIV-FAM/BHQ with FAM as a reporter dye located at the 5′ end and a black hole quencher at the 3′ end, 5′-TGC CCG TCT GTT GTG TGA CTC TGG TAA-3′ (10 μM), 5 μl cDNA, and RNase free water to a final 25 μl reaction volume. qRT-PCR thermocycling conditions were as follows: incubation (50°C, 2 min), initial denaturation (95°C, 10 min), and 44 cycles of denaturation (95°C, 30 s) and annealing and extension (60°C, 1 min). Quantitation of cDNA was done relative to triplicate standard curves generated in each run from serial dilutions of a plasmid containing a viral DNA insert. Three "no template" controls were included for each run.

$CD4^+$ T cell counts

A single platform technique (SPT) was used to enumerate $CD4^+$ T-helper cells using BD TruCount tubes (BD Biosciences, San Jose, CA). Of EDTA whole blood 50 μl was stained using 5 μl monoclonal antibody mixture BD TriTEST CD3-FITC/CD4-PE/CD8-PerCP (BD Biosciences) followed by 450 μl 1 × BD lysis and fixative solution. Data acquisition and analysis by the MultiTEST software were performed using a three-color BD FACS Calibur (Becton Dickinson Immunocytometry Systems 2350).

Data analysis

Prevalence values were calculated as the proportion of positives per all tested subjects. Differences between means were calculated using the Student's t test. All analyses were done using Stata version 10 (StataCorp LP).

Results

Baseline characteristics

Our descriptive study was conducted in treatment-naive patients recruited during two time periods: in 2005–2007, representing the first years of operation of the KIULARCO cohort, and in 2009, 4 years after the initial sampling period. The clinical and demographic characteristics of the 120 patients from 2005–2007 and of 119 patients from 2009 are summarized in Tables 1 and 2. We failed to amplify PCR products from 36% and 40% of samples of periods 2005–2007 and 2009, respectively. Lack of amplification in such a high proportion of samples was probably due to poor long-term storage of the samples in Tanzania, likely resulting in degradation of viral RNA. The median age of the study participants was 35 years in 2005–2007 and 40 years in 2009. The percentage of female study participants was 68.4 in 2005–2007 and 62.3 in 2009. In baseline samples from 2005–2007 the median CD4 cell count was 222 cells/μl and the median viral load was 50,805 copies/ml, whereas in 2009 the median CD4 cell count was 420 cells/μl and the median viral load was 39,920 copies/ml.

During the first years of our HIV cohort several parameters were added or modified after incorporating improvements in patient diagnosis or due to new technical capabilities in the laboratory. Associated with this improved patient care the center also experienced earlier recruitment into the cohort. Due to these contextual differences in sampling for groups 1 and 2, we investigated the comparability of both sample sets. One identified major difference was the mean time to ART initiation, which tended to be significantly shorter in the later period. To compensate for this we compared for 2005–2007 the prevalence of single nucleotide polymorphisms associated with drug resistance (DR-SNPs) in 75 samples immediately after ART initiation with 44 samples from delayed ART initiation. In addition, we considered WHO stage, age, sex, subtypes, means of CD4 counts, and viral load values. Of all parameters tested, only the difference in mean age between both comparison groups was significant (95% confidence interval, 0.5–9.5, $p = 0.0396$). Since there were no significant

Table 1. Comparison of Baseline Categorical Characteristics of KIULARCO Patients in 2005–2007 and 2009

Variable	Years 2005–2007 Frequency	Proportion	95% CI	Year 2009 Frequency	Proportion	95% CI	Difference in proportion 95% CI (p)
WHO stage		(*n* = 116)			(*n* = 118)		
1	39	0.34	±0.09	60	0.51	±0.09	0.05–0.30 (*p* = 0.0084)[a]
2	22	0.19	±0.07	35	0.3	±0.08	0.00–0.22 (*p* = 0.0678)
3	35	0.30	±0.08	21	0.17	±0.07	0.012–0.23 (*p* = 0.0319)[a]
4	20	0.17	±0.07	3	0.03	±0.03	0.07–0.22 (*p* = 0.0001)[a]
Sex		(*n* = 117)			(*n* = 118)		
Females	80	0.68	±0.08	74	0.63	±0.09	−0.07–0.18 (*p* = 0.4108)
DR mutations		(n = 120)			(n = 119)		
Major RTIs	10	0.08	±0.05	4	0.03	±0.03	−0.01–0.11 (*p* = 0.1069)
Major NRTIs	4	0.03	±0.03	1	0.01	±0.02	−0.01–0.06 (*p* = 0.2128)
Major NNRTIs	9	0.08	±0.05	4	0.03	±0.03	−0.02–0.10 (*p* = 0.1666)
Minor RTIs	3	0.03	±0.03	6	0.05	±0.04	−0.02–0.07 (*p* = 0.4994)
Minor PIs	16	0.13	±0.06	7	0.06	±0.04	0.00–0.15 (*p* = 0.0509)
Subtypes[b]		(*n* = 120)			(*n* = 111)		
A1	38	0.31	±0.09	30	0.27	±0.08	−0.07–0.17 (*p* = 0.4395)
C	52	0.43	±0.09	49	0.44	±0.09	−0.11–0.14 (*p* = 0.9013)
D	22	0.18	±0.07	15	0.13	±0.06	−0.05–0.09 (*p* = 0.3183)
A1, C	3	0.03	±0.02	2	0.02	±0.02	−0.07–0.14 (*p* = 0.7156)
A1,D	0	0	0	2	0.02	±0.02	−0.02–0.07 (*p* = 0.1397)
C,D	3	0.03	±0.02	8	0.07	±0.05	−0.02–0.12 (*p* = 0.0933)
A1,01_AE	2	0.02	±0.02	3	0.03	±0.05	−0.04–0.07 (*p* = 0.5888)
A1,C,D	0	0	—	1	0.01	±0.02	−0.03–0.06 (*p* = 0.2974)
A1,01_AE,C	0	0	—	1	0.01	±0.02	−0.03–0.06 (*p* = 0.2974)
All recombinants	8	0.07	±0.04	17	0.15	±0.06	−0.01–0.17 (*p* = 0.0345)[a]

[a]Statistically significant.
[b]Subtype assignment according to prediction by Los Alamos database.
CI, confidence interval; DR mutation, drug resistance mutation; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PIs, protease inhibitors.

differences in DR-SNPS or other factors when comparing samples with early versus late treatment start (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/aid), all 2005–2007 samples were pooled for comparative analysis with the 2009 data set. Based on this comparison we concluded that variation in the time to ART initiation did not introduce a major sampling bias.

Prevalence of HIV-1 subtypes

HIV-1 subtypes were determined from the reverse transcriptase sequence (codon 23 to 236). Subtype frequencies are shown in Fig. 1 for both study periods. Subtype C was found to be the most prevalent HIV-1 subtype with a frequency of 43% for the years 2005–2007 and 44% for 2009. Other subtypes and their frequency of occurrence during our sampling periods were A1 (32% for 2005–2007 and 27% for 2009), D (18% and 13%), and recombinants (7% and 15%), respectively (Table 1). The phylogenetic relationship of the sequences from 2005–2007 and 2009 is shown in Supplementary Figs. S1 and S2. The corresponding GenBank accession numbers of these sequences are KC537065–KC537290.

Prevalence of antiretroviral resistance mutations

The major and minor drug resistance mutations in 2005–2007 and 2009 and their respective frequencies (number of observed cases in each group of samples) are shown in Tables 3 and 4 and Fig. 2. The frequency of observed mutations was high overall, but several of these mutations were found to

Table 2. Comparison of Baseline Numeric Characteristics in 2005–2007 and 2009 in KIULARCO Patients

Variable	CD4 counts (cells/μl) 2005–2007	2009	Viral load (copies/ml) 2005–2007	2009	Age (years) 2005–2007	2009
Median	222	420	50,805	39,920	35	40
95% confidence interval	174–270	359–481	32,070–69,540	9,557–70,283	34–36	38–42
Range	3–1,298	20–2,079	35–1,270,126	1,380–2,417,380	4–85	21–71
Interquantile range (25–75)	236–365	169–310	80,352–146,791	71,770–304,209	8.5–15.5	9.3–16.7
Sample size	117	118	90	63	117	120
Difference between medians	198		10,885		5	
95% CI of difference between medians	119–277		23,594–45,364		2.7–7.3	

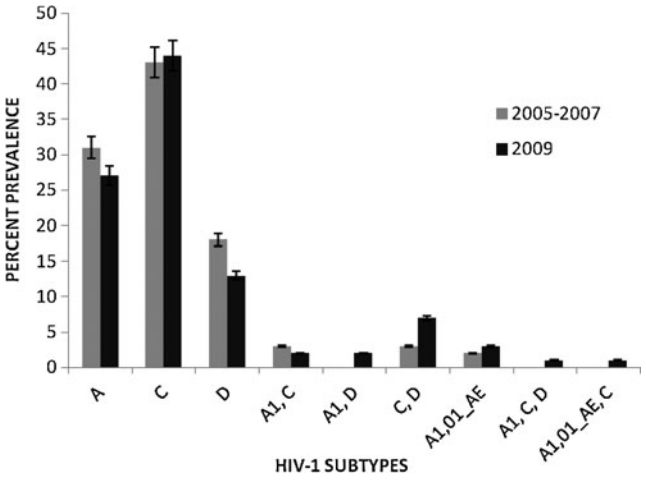

FIG. 1. Prevalence of HIV-1 subtypes in the Ifakara HIV-1 cohort in 2005–2007 and 2009. Bars represent 95% confidence intervals. Subtype assignment according to prediction by the Los Alamos database.

cluster in few individuals. Thus from these mutation frequencies the prevalence rate of major reverse transcriptase inhibitor mutations in the baseline samples from individuals in 2005–2007 was 8.4%, and, quite unexplained, the prevalence was lower at 3.3% in 2009. Nucleoside reverse transcriptase inhibitor (NRTI) mutations occurred in 3.4% and 0.8% of all tested individuals in 2005–2007 and 2009, respectively, while the more prevalent nonnucleoside reverse transcriptase inhibitor (NNRTI) mutations were 7.6% and 3.3% for the same periods. Generally we observed a reduction in DR mutations (particularly for K103N and Y181C) for HIV-1 subtype C from 2005–2007 to 2009 (Supplementary Table S3).

Table 3. Major HIV-1 Drug Resistance Mutations in Treatment-Naïve Ifakara Patients in 2005–2007 and 2009

Year	*Patient ID*	*Subtype*[a]	*Major DR-SNPs*	*Sex*	*Age (years)*
2005–2007	5410033	D	K103N	F	42
	5510060	D	M41L	F	40
	5510135	C	K103N	M	47
	5510184	A1	M184V, G190A	F	29
	5510187	C	K103N, Y181C	F	4
	5510259	C	K103N	F	30
	5511494	C	K103N	F	30
	5510599	C	Y181C	F	51
	5510039	A1	M184V, G190A	F	31
	5510072	C	K65KR, K70KR, L74LV, M184V, K103N, Y181C	F	45
2009	5514370	A	K103N, M184V, T215F	F	63
	5421041	A1	K103N	—	—
	5513670	C	K103N	M	35
	5511139	C	K103N	M	4

[a]Subtype prediction according to Stanford HIV-1 Drug Resistance database, version 6.2.0.

DR-SNPs, drug resistance single nucleotide polymorphisms.

Table 4. Frequency of Observation of HIV-1 Drug Resistance Single Nucleotide Polymorphisms in KIULARCO Cohort in 2005–2007 and 2009

DR-SNP	*2005–2007 (n=120) Frequency (%)*	*2009 (n=119) Frequency (%)*
Major NRTI mutations		
M41L	1 (0.8)	—
K65KR	1 (0.8)	—
K70KR	1 (0.8)	—
L74LV	1 (0.8)	—
M184V	2 (1.6)	1 (0.8)
M184MV	1 (0.8)	—
T215F	—	1 (0.8)
Subtotal: major NRTI	7 (5.9)	2 (1.7)
No. of individuals with major NRT mutations	4 (3.4)	1 (0.8)
Major NNRTI mutations		
K103N	6 (5.0)	4 (3.3)
Y181C	3 (2.5)	—
G190A	2 (1.7)	—
Subtotal: major NNRTI	11 (9.2)	4 (3.3)
No. of individuals with major NNRT mutations	9 (7.6)	4 (3.3)
Total major RT mutations	18 (15.1)	6 (5.0)
No. of individuals with major RT mutations	10 (8.4)	4 (3.3)
Minor NRTI mutations		
M41KM	1 (0.8)	—
D67G	—	1 (0.8)
D69S	1 (0.8)	—
V118I	—	3 (2.5)
H221HY	1 (0.8)	—
Subtotal: minor NRTI	3 (2.5)	4 (3.3)
Minor NNRTI mutations		
V90I	—	1 (0.8)
V108I	—	1 (0.8)
V179D	—	1 (0.8)
G190R	1 (0.8)	—
Subtotal: minor NNRTI	1 (0.8)	3 (2.5)
All minor mutations in RT	4 (3.4)	7 (5.8)
Minor protease mutations		
L33FL	—	1 (0.8)
L10V	9 (7.6)	1 (0.8)
L10I	4 (3.4)	1 (0.8)
L10IL	—	1 (0.8)
L10IV		1 (0.8)
V11I	2 (1.7)	—
V11IV	—	1 (0.8)
L23F	1 (0.8)	—
A71T	1 (0.8)	—
T74S	1 (0.8)	—
L89V	—	1 (0.8)
Total: minor protease mutations	18 (15.1)	7 (5.8)

DR-SNPs, drug resistance single nucleotide polymorphisms; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor.

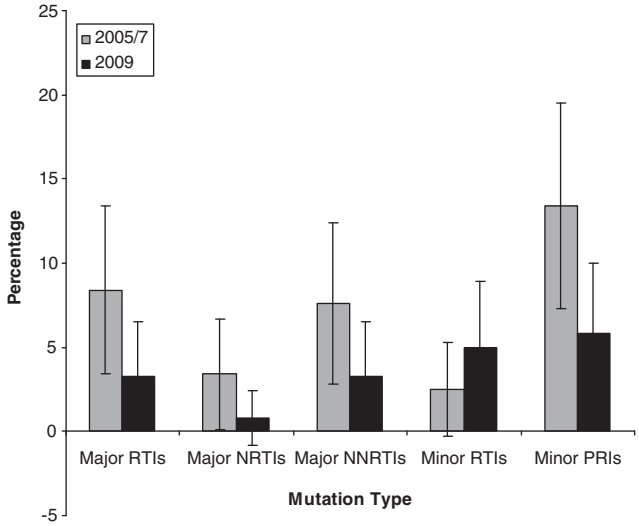

FIG. 2. Prevalence of HIV-1 drug resistance (DR) mutations in 2005–2007 and 2009 in the Ifakara HIV-1 cohort. RTIs, reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PIs, protease inhibitors. Bars represent 95% confidence.

In subtype C viruses the prevalence of K103 was 4.2% for the period of 2005–2007 while in 2009 it was 1.7%. In addition, the prevalence of the Y181C mutation was 2.5% for subtype C viruses in 2005–2007, whereas it was not found at all in our samples from 2009. No major protease gene mutation was found in both study periods.

Discussion

The work at the CDCI and subsequently the KIULARCO cohort in rural Tanzania was initiated at the time of commencing the ART rollout in these two districts. Close clinical monitoring of patients was installed for guiding treatment and adaptation of drug regimens. The comprehensive data continuously gathered for this rapidly growing cohort also provided the basis for epidemiological studies. A central question was to what extent the deployment of ART would drive the development of drug resistance under the specific conditions of a rural African setting. As in other areas with limited resources, a number of typical shortfalls can contribute to treatment failures, e.g., incomplete adherence to treatment, limitations in travel capabilities for patients for their medical appointments or drug pickup, or stock out of drugs at health institutions. Despite little prior availability of ART in the study area some primary resistances, i.e., new infections with a drug-resistant strain, had to be expected to occur according to reports from other areas in Tanzania.[12,16] In Tanzania the prevention of mother-to-child transmission (PMTCT) of HIV-1 policy (2009) recommends combination regimens that include zidovudine (AZT), nevirapine (NVP), and lamivudine (3TC) in areas with the capacity to offer and monitor ART. For all other areas with no such capability the policy recommends only the use of minimum antiretroviral prophylaxis consisting of a single dose of nevirapine to the mother and the infant.[8] Particularly in the Ifakara area the use of single dose nevirapine for PMTCT has been practiced since 2004 (Marcel Stoeckle, personal communication). This policy with less than perfect drug pressure may have contributed to the development of viral resistance to nevirapine and hence to the transmission of the drug-resistant HIV-1 strains.[17]

During the first 3 years of KIULARCO (2005–2007), about 20 cases of suspected resistance had been observed. This assumption was based on clinical parameters or CD4 decline in the absence of any hint of poor patient's adherence. The main driving parameters could have been that in this early phase of the cohort much of the cost of medication had to be covered by the patients and distribution was via private pharmacies (Marcel Stoeckle, personal communication). This prompted our investigation of the transmission of primary resistance in the study area. In an attempt to gather baseline data for molecular epidemiological studies on drug-resistant HIV-1 infections among KIULARCO patients, we compiled two molecular data sets, one describing the HIV-1 diversity and subtypes in the study area and the other providing information on the prevalence of DR-SNPs in treatment-naive patients. Because prior to a rollout of ART the transmission of resistant virus strains is likely a rare event, we expected to find very few DR-SNPs in treatment-naive individuals.[18]

HIV-1 subtypes C, A, and D and the recombinant forms were the most frequent ones identified in KIULARCO participants. Despite slight differences in the proportion of these subtypes, this distribution is overall in line with other reports from different regions of Tanzania.[1] In studies conducted in the Dar es Salaam, Kilimanjaro, Kagera, and Mbeya regions, subtypes A and C were also found to be the predominant HIV-1 subtypes.[13,19] Our finding of a higher proportion of subtype C in Ifakara is plausible, since Ifakara is directly linked by railway to Zambia, where subtype C prevails.[20]

Our analysis is representative in the context of other studies as the *pol* region has been used previously for subtyping HIV-1.[1,19] This approach represents a straightforward way of identifying circulating HIV-1 subtypes in population studies, especially in resource-poor settings. As evidenced by the uncertainty about subtype A1 versus CRF01_AE, a limitation of our subtype analysis may, however, lie in the restriction to HIV-1 protease and reverse transcriptase as these sequences might not capture the full diversity and may underestimate/misclassify some of the recombinant forms.[13]

Overall our findings highlight the diversity of HIV-1 subtypes in rural Tanzania and contribute further data points to a countrywide picture of subtype frequencies. The longitudinal comparison indicates stable subtype frequencies over a period of at least 4 years with only significant changes in the proportion of recombinants, whereby more recombinants were observed in 2009 than in 2005–2007 ($p = 0.0345$).

We compared the prevalence of DR-SNPs in drug-naive patients in two surveys, conducted in 2005–2007 and 2009. The rationale was to describe potential effects of ART rollout on the prevalence of DR-SNPs. The first sampling period 2005–2007 was chosen closely after the beginning of the Tanzanian ART program (2004) and the second group of samples was collected during 2009. We assumed that the effects of drug pressure might lead to a first visible increase in transmitted resistance mutations in the population during subsequent years.

When comparing the two groups of patients, the significant difference in the prevalence of drug-resistant mutations may suggest a sampling bias in the sampled patients, e.g., by

sampling in distinct populations with different levels of drug exposure/experience. Of note, the first analysis period 2005–2007 falls into the early rollout period. During this time no drug distribution or only standardization in drug distribution was established (mostly through private pharmacies), and many of the patients had to pay for the drugs. These two factors certainly introduced great uncertainty and possibly suboptimal drug dosing as it largely depended on the patients' financial situation. For the latter time period (2009) government-driven drug distribution programs made the medication freely available for the patients rendering the likelihood of continuous drug availability for the patient higher.

Between our study periods also the context of patient recruitment might have differed. We studied treatment-naive patients from two time periods: 2005–2007, representing the initial years of the KIULARCO cohort and of drug rollout, and about 4 years after this period. As expected, we found some differences in baseline characteristics between our two groups, e.g., in median CD4 counts and viral loads. Differences in the time to initiation of ART could explain the observed discrepancy. To probe whether these differences were relevant for our research question, we analyzed in parallel samples from patients with immediate ART initiation versus later initiation and found that both subgroups of 2005–2007 samples were very comparable without significant differences with respect to the prevalence of DR-SNPs. This prompted us to analyze in 2009 samples from treatment-naive individuals irrespective of the time point at which these study participants started ART. Therefore we cannot rule out that a discrepancy in the time to ART initiation has contributed to the observed differences (samples of 2005–2007 had slightly lower median CD4 counts and higher median viral loads than the 2009 group), despite our result indicating comparability between the two subsets from 2005–2007.

When comparing the prevalence of major reverse transcriptase mutations for 2005–2007 versus 2009 we found a higher prevalence (8.4%) in samples from earlier years than in 2009 (3.3%). This difference in prevalence was not statistically significant ($p = 0.1069$). The prevalence of nonnucleoside reverse transcriptase mutations was 3.4% in 2005–2007 and 0.8% in 2009, respectively ($p = 0.2128$). The higher rate of mutations in earlier years might reflect the specific context of sampling right at the beginning of ART rollout in the region. Of note, more patients in 2005–2007 than in 2009 were judged as WHO clinical stages 3 and 4. Thus more patients at WHO clinical stages 3 and 4 were enrolled in the KIULARCO cohort at the start of the Tanzanian ART program. And we might expect a higher prevalence of DR-SNPs after the extended treatment period of 5 years of ART in the Ifakara cohort. However, we observed that over time the number of DR-SNPs had rather decreased (2009 vs. 2005–2007). We believe that the initial therapy situation provides a most plausible explanation. Before 2007 the drug distribution system was not yet well established, and many patients had to pay for their drugs. In low-income areas of our study this will have inevitably led to situations of suboptimal drug availability and irregular drug dosing and is likely seen in our figures, which reflect the improving national HIV treatment management system in Tanzania.

The NNRTI mutations K103N, Y181C, and G190A cause high-level resistance to nevirapine, one of the most common NNRTI drugs used in Ifakara and elsewhere in Tanzania.[21,22] All individuals with DR-SNPs in 2009 and 60% of individuals in 2005–2007 carried the K103N mutation. The high prevalence of the K103N mutation correlates with the use of nevirapine monotherapy for PMTCT in Tanzania.[8] And the preexistence of this mutation likely influences the outcome of ART by allowing an early start for the further accumulation of DR-SNPs under suboptimal drug pressure. KIULARCO and other Tanzanian ART programs can offer only few treatment options.[21] The observed level of primary resistance must therefore be considered a potential threat to the effectiveness of the current Tanzanian ART program.[17,22]

In summary, our study identified DR-SNPs in drug-naive patients during both periods, immediately after ART introduction in Tanzania and 4–5 years after. We identified a trend toward lower mutation frequencies in samples collected later in the cohort. Minor differences in the repertoire of mutations might reflect individual introductions from abroad, a finding due to the small sample size. The overall number of patients in the KIULARCO study under ART was 1,491 in December 2008.[14] These numbers might still be too small to describe any stable trends in transmitted resistance mutations within 4 years, but the observed findings will be followed-up in the coming years.

Overall our findings are in agreement with genotypic results from other African studies and confirm that primary drug resistance mutations are present in treatment-naive patients even before the scale up of ART.[12,16]

Conclusions

Our molecular typing provides the baseline information on HIV-1 strains identified in a rural area in southern Tanzania. Our data support a stable subtype distribution and an overall low prevalence of preexisting drug resistance. Monitoring the extent and significance of HIV-1 drug resistance mutations in treatment-naive individuals is and will continue to be a key for an informed choice of optimal ART and thus can contribute to efforts toward preventing the spread of HIV-1 drug resistance.

Sequence Data

The GenBank accession numbers for the RT sequences generated in this work are KC537065–KC537290.

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Author Disclosure Statement

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